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The Biologically Active Biopolymer Silk: The Antibacterial Effects of Solubilized *Bombyx mori* Silk Fibroin with Common Wound Pathogens

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Antibacterial properties are desirable in wound dressings. Silks, among many material formats, have been investigated for use in wound care. However, the antibacterial properties of liquid silk are poorly understood. The aim of this study is to investigate the inherent antibacterial properties of a *Bombyx mori* silk fibroin solution. Silk fibroin solutions containing ≥ 4% w/v silk fibroin do not support the growth of two common wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. When liquid silk is added to a wound pad and placed on inoculated culture plates mimicking wound fluid, silk is bacteriostatic. Viability tests of the bacterial cells in the presence of liquid silk show that cells remain intact within the silk but could not be cultured. Liquid silk appears to provide a hostile environment for *S. aureus* and *P. aeruginosa* and inhibits growth without disrupting the cell membrane. This effect can be beneficial for wound healing and supports future healthcare applications for silk. This observation also indicates that liquid silk stored prior to processing is unlikely to experience microbial spoilage.

1. Introduction

Wound care is a key area of healthcare and can have long-lasting adverse effects on quality of life if wound infection is not treated correctly by medical professionals. Wound healing typically follows a pathway consisting of four clear stages: hemostasis, inflammation, proliferation, and remodeling[1]; however, this order is disrupted in chronic wounds, making treatment of these wounds an especially difficult challenge. The inflammation stage can also be prolonged by contamination by microbial pathogens present in the environment, the surrounding skin, or various mucous membranes.[2]

Two of the most common pathogens that cause wound infection are the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[3,4] *S. aureus* is a gram-positive bacterial species that can grow aerobically or facultatively anaerobically[5] and is found in the environment and on human skin and mucous membranes as part of a healthy skin flora. However, this microbe can cause serious infections if it enters the bloodstream or internal tissues. *P. aeruginosa* is a gram-negative motile bacterium found on the skin, in the throat, and in stool samples of healthy individuals. However, *P. aeruginosa* can become pathogenic by the release of virulence factors, such as pyocyanin. *P. aeruginosa* can form intractable biofilms, which make subsequent wound treatment difficult.[6,7] Wound dressings are a commonly used method for preventing infection and providing an environment that favors wound healing. Our ability to unspin the silk fiber and generate novel material formats[8,9] has sparked a renewed interest in silk, including its use in wound care applications (e.g., refs[10–12], reviewed ref.[13]).

Bombyx mori silk has long been used in humans for wound care, especially as a suture material, and the *B. mori* silk fibroin protein is a clinically approved biomaterial for use in load-bearing applications in humans (e.g. sutures, meshes, etc.).[14] Silk fibroin films, sponges,[15] hydrogels,[16] and knitted scaffolds[11] have also shown potential uses in wound repair and aesthetic restorations in small-scale clinical trials. Soluble silk has also improved epithelial healing in a rabbit eye cornea model.[12,17]

Overall, silk fibroin is emerging as a useful tool in wound healing, but wound dressings should help to prevent or overcome pathogen infection in wounds. Although some research studies have focused on a potential role for sericin, one of the components of raw silk is exposed to the elements,[18] and the results have been conflicting,[19–22] reviewed in ref.[23]. For

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example, the ability of raw silk fibers from silkworm cocoons to provide a substrate for limited bacterial biofilm growth was thought to protect the developing silk moth from environmental stresses.[19,24] Several studies have examined silk as it is spun in the cocoon, but others have investigated it when processed into silk sutures or scaffolds.[19,24] Still others have explored silk films cast from regenerated silk fibroin and have found strong microbial attachment, thought to be mediated by the glycine, alanine, and serine amino acids present in silk fibroin.[25] Silk fibroin processing has also been linked to antibacterial effects, especially in relation to the solubilizing agents lithium bromide and Ajisawa's reagent. Silk films cast from lithium bromide-solubilized silk showed a higher degree of antibacterial activity.[26] Blending silk fibroin with polyethyleneimine,[27] graphene oxide,[28] or plant extracts,[29] has also been explored to improve antibacterial effects. Overall, more research is needed to characterize the antimicrobial properties of silk fibroin for use in wound dressings or other formats.

Healthcare products, like wound dressings, that are destined for use in clinical applications typically must also be sterile. However, with silk fibroin, this requirement introduces new challenges because the sterilization methods that are deemed acceptable by regulatory agencies can adversely affect this biomaterial.[30–33] Current methods of sterilization include autoclaving, irradiation (e.g. ultraviolet, gamma), immersion in 70% v/v ethanol, filtering, or exposure to ethylene oxide. Some studies have indicated that autoclaving a silk solution decreases molecular weight, increases protein aggregation, and ultimately affects the final product by altering the physical properties of the silk.[34] Filtering a silk solution through a 0.22 μm filter is only feasible with low molecular weight silk (i.e., low viscosity solutions) and at low concentrations to minimize losses in sample concentration and final volume.[30]

A knowledge gap presently exists regarding the sterility of silk fibroin solutions and the effects of silk fibroin on bacterial growth. One aim of the present work was to produce sterilized and non-sterilized silk solutions and to assess their sterility over time under various storage conditions. A second aim was to use live/dead bacterial staining and colony counting to assess the viability of *S. aureus* and *P. aeruginosa* after inoculation into a silk fibroin solution. The final aim was to mimic wound conditions using nutrient agar to determine whether adding silk fibroin-soaked wound pads would eliminate bacterial growth on the agar plates.

2. Results and Discussion

2.1. Pure Aqueous Silk Solution

The dialysis progress was monitored using conductivity measurements of the dialysate, which served as a proxy (**Figure** 1A). At the first water change, the conductivity was 4863.50 ± 335.40 μS cm^{-1} (n = 4), and the conductivity value dropped at each water change. For example, at the start of day 2, the conductivity was 588.5 ± 93.54 μS cm^{-1} and dropped to 14.75 ± 10.21 μS cm^{-1} by the end of the day. The final conductivity was 9 ± 2.45 μS cm^{-1}, which is less than the conductivity of tap water (50 μS cm^{-1}). This evaluation is important because

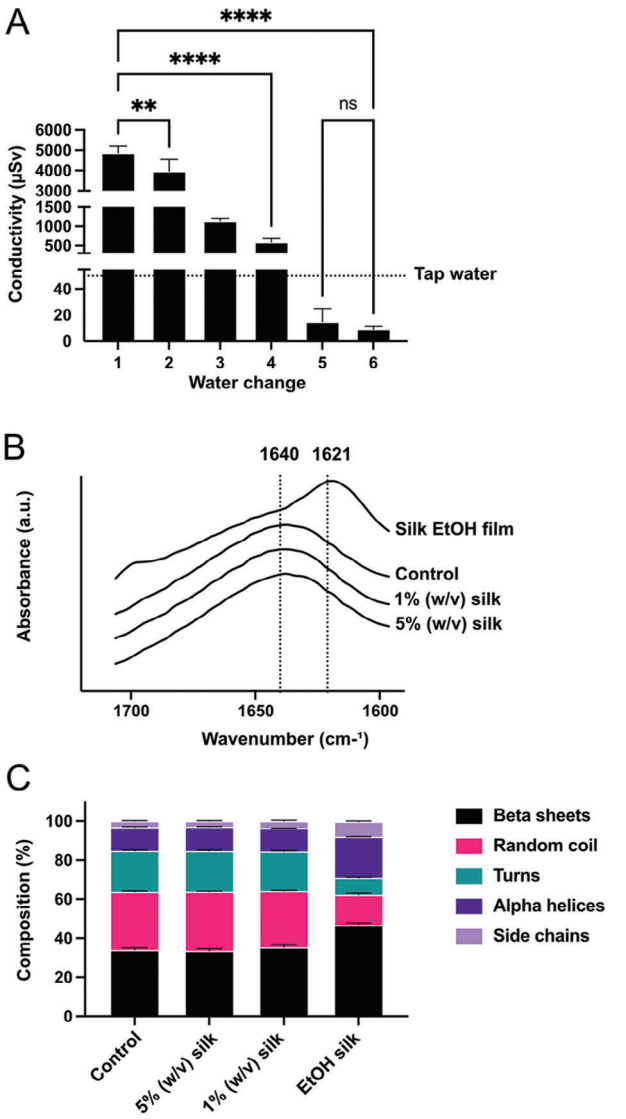


Figure 1. Silk fibroin solution characterisations. A) Conductivity measurements of the dialysate over 72 h resulting in an aqueous silk fibroin solution. B) FTIR spectra and C) FTIR analysis of silk fibroin solution incubated in the orbital incubator at 37 °C 150 rpm for 24 h. Silk was air dried into films for analysis. Airdried amorphous control and ethanol (EtOH) treated crystalline silk film controls; the crystalline control data are reproduced with permission.[10] Data analysis evaluated by one-way ANOVA followed by Tukey's multiple comparisons test. Asterisks denote statistical significance determined using post-hoc tests as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ($n = 3$, ± SD)

the reverse engineering of silk fibers into a silk fibroin solution requires that the silk molecule be dissolved to dismantle higher-order structures.

Previous studies have shown that the choice of silk fibroin extraction protocol can influence the antibiotic activity of the resulting silk films.[26] However, the degree to which this reported effect can be attributed to contamination remains to be determined. For example, in the present study, we used a 9.3 M lithium bromide (LiBr) solution to disrupt the robust beta-sheet secondary structures. Therefore, residual LiBr could impact bac-

terial viability. Another chemical utilized in reverse engineering silk fibers is sodium carbonate, which can also have bactericidal effects.[35] The sodium content was verified to be at the expected 0.02 M level before silk degumming and it increased to 0.021 M after boiling. Washing the degummed silk fibers with water and analyzing the washing fractions for their sodium carbonate content showed concentrations starting at 0.9 ± 0.2 mM on rinse 1, dropping to 6 ± 4 μM on rinse 2, and plateauing at 0.2 ± 0.06 μM by the final rinse. The concentration of sodium in the final silk fibroin solution (5% w/v) was estimated to be 50 ± 3 μM (Figure S3A, Supporting Information). In the present work, the reduction in conductivity to baseline levels suggests that LiBr was quantitatively removed. By contrast, the measurements with the sodium ion selective electrode suggested that some sodium was still present in the silk fibroin solution after reverse engineering. The presence of these residual sodium ions could have had a small bactericidal effect (Figure S3B, Supporting Information), but the residual level (50 μM) was well below the level (5 mM) that would have completely blocked bacterial growth. Instead, we observed a complete block of bacterial growth by the prepared silk fibroin (detailed below). Therefore, the effects observed here can be attributed to the action of silk fibroin itself, rather than residual contaminants.

2.2. Secondary Structure Analysis of Silk

For some of the microbial assays, we exposed silk fibroin to shear forces and elevated temperatures. We therefore felt that assessing possible structural changes was important. We used FTIR to determine the secondary structures of silk fibroin in 1 and 5% w/v solutions before and after incubation in the orbital incubator at 37 °C, 150 rpm (Figure 1B,C). Samples of silk solution were cast and airdried to form silk films. Amorphous control silk films were produced from silk fibroin solution stored at 4 °C. The most common secondary structures were detected in the following order: beta sheets > random coils > turns and > alpha helices. Deconvolution showed that the beta sheets in the amorphous control, 1 and 5% w/v silk films were 33.91 ± 1.14, 33.43 ± 1.19, and 35.21 ± 1.43%, respectively. No statistically significant differences were noted in beta-sheet content in the amorphous control films ($p = 0.6572$ for 5% silk and $p = 0.0601$ for 1% silk). The other secondary structures also showed no significant differences. By contrast, the ethanol-treated silk film showed an increase in crystalline beta sheets (46.61% vs 33.91%).

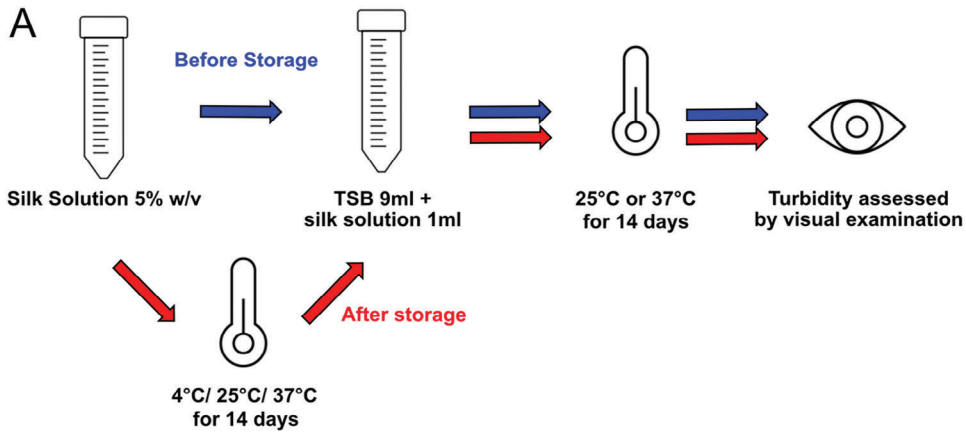
Elevated temperatures and shearing forces can trigger structural changes in an amorphous silk solution.[36] Therefore, monitoring the impact of incubation and shear on secondary structure was also deemed important. In the case of secondary structure changes, we expected a higher percentage of beta sheets and a reduced percentage of amorphous structures. No conformational changes were observed, indicating that the crystallinity of the silk fibroin solution was unaffected by incubation at 37 °C and rocking at 150 rpm for 24 h. This confirms that we are detecting the bactericidal effects of aqueous amorphous silk solution.

Next, we wanted to probe the solution conformation of silk using pyrene labeling. Pyrene can detect polarity changes in the environment and this, in turn, can be detected as changes in the emission peaks when excited at 336 nm.[37,38] Specifically, pyrene is characterized by five monomer bands, with the band at 385 nm being particularly useful for analyzing protein conformation. The ratio of these bands can be used to determine the "Py value". Therefore, we used the intensity ratio of 385 to 375 nm to monitor silk hydrophobicity (Figure S6a, Supporting Information). The Py values for the silk samples were significantly different from the water control and indicated a change to a hydrophobic condition (Figure S6b, Supporting Information). The significant difference between the 2% and 4% (w/v) silk solution samples was small, but the silk samples overall had similar values, suggesting that no changes in solution conformation had occurred at the tested silk fibroin concentrations. This is in line with previous work that reported a critical micelle concentration for silk fibroin as 0.4 mg mL^{-1}.[37]

2.3. Sterility of Aqueous Silk Fibroin

Aqueous protein solutions typically provide a preferred environment for bacterial growth over protein in its dry state. We therefore examined the susceptibility of liquid silk to bacterial spoilage. For our studies, 9 mL tryptic soya broth (TSB) was spiked with a 1 mL silk fibroin 5% w/v solution. This silk fibroin solution was exposed to various processing conditions (**Figure 2A**). First, freshly prepared silk fibroin solution samples were assessed either "as-obtained" or after filter sterilization. When fresh as-obtained silk fibroin solution was added to TSB and incubated at either room temperature or 37 °C, turbidity was observed in the solutions at the end of this incubation period. This suggested that the freshly prepared silk solution was contaminated with bacteria (Figure 2, "Before storage" column). However, turbidity was observed only after 14 days of incubation, suggesting that the bacteria were present at initially low numbers and/or were slow-growing. The absence of cloudiness in the filter-sterilized silk solution demonstrated the effectiveness of this process for bacterial cell removal (Figure 2, "Before storage" column). The turbidity of the TSB was assessed and verified with control experiments (Figure S4, Supporting Information). Specifically, TSB was spiked with a low number of bacterial cells (ranging from 10^1–10^7 CFU mL^{-1}). Immediate inspection of these spiked samples revealed no visible turbidity. However, after a 24 h incubation, turbidity was clearly visible at all tested bacterial concentrations. Overall, this control experiment demonstrated that we were able to detect low levels of contaminants within 24 h.

We also exposed sterile-filtered and as-prepared silk fibroin solutions to different temperature storage conditions (4 °C, room temperature (≈25 °C) or 37 °C) for 14 days. We then used the stored silk samples to spike TSB and incubated the spiked solutions for 14 days at either room temperature or 37 °C. Interestingly, no turbidity was observed in any of these samples (Figure 2, "After storage" column), suggesting that, although the silk solution was contaminated during preparation, the culture environment was not conducive to bacterial growth and/or survival. Therefore, bacterial growth was only seen before storage of the silk solution, suggesting that bacteria are likely introduced dur-



B

		Before storage		After storage	
		TSB Incubation for 14 days		TSB Incubation for 14 days	
	Silk Storage temp	25°C	37°C	25°C	37°C
Filtered	4°C				
	25°C				
	37°C				
Unfiltered	4°C				
	25°C				
	37°C				

No growth
Growth

Figure 2. Sterility testing of silk fibroin solution (5% w/v) in tryptic soya broth (TSB) before and after storage at 4 °C, room temperature and 37 °C for 14 days. A) TSB was incubated at both room temperature and at 37 °C for 14 days and then examined for growth (i.e. turbidity) and B) recorded. Green and yellow color coding indicates no growth and growth, respectively. ($n = 3$)

ing silk solution processing but their viability is not supported during storage.

Besides the TSB we also used agar to identify any colonies that would grow. The sterility of the silk solutions was confirmed by streaking samples of the sterilized and non-sterilized silk fibroin solution, before and after storage for 14 days, onto blood and chocolate agar plates. These plates were incubated either aerobically or anaerobically before the final assessment. These enriched agar growth media are formulated for the isolation of pathogenic bacteria.[39] The agar plates showed no growth when plated with silk fibroin before or after storage, broadly supporting the TSB results. The finding of samples that showed bacterial contamination with the TSB method suggested that growth was slow and required the nutrients supplied by TSB (and not available in the agar plates). Overall, these results suggest that the regenerated silk fibroin solution has an inherent ability to attain and maintain sterility for 2 weeks. This is an interesting discovery because many sterilization techniques adversely affect the silk molecule (through molecular weight reduction, polydispersity, etc.) and the silk solution properties (e.g. premature self-assembly).[30,32,40,41]

2.4. Minimum Inhibitory Concentration for Silk

Next, we determined the minimum inhibitory concentration for liquid silk because aqueous liquid silk fibroin exhibits antibacterial properties. We also conducted control experiments to exclude possible confounders, especially the impact of water on bacterial colony count. Colony counts in water and Luria Bertani Lennox (LB) served as benchmarks for low and high growth conditions, respectively. Water controls were important because an aqueous silk fibroin solution was spiked with pathogens to determine its minimum inhibitory concentration (Figure S2, Supporting Information). Specifically, we examined the impact of water on the bacterial strains *S. aureus* and *P. aeruginosa* because these are common wound pathogens[3,4] (Figure S2, Supporting Information). The contents of sterile water added were 75%, 85%, 95%, and 100% of the total volume, with the remaining volume comprising LB media to control for the presence of nutrients. Water was spiked with the pathogens, and colony counting was performed before and after 24 h incubation. No significant difference was observed in bacterial growth in 75–100% water compared to the 100% LB control with *S. aureus*, which showed a significant increase ($p = 0.0054$). As compared to spiked 100% water, we observed a significant increase in growth in 75% water with *P. aeruginosa* ($p = 0.0377$) and a more significant increase in LB media ($p = 0.0041$); however, we did not observe any significant change in bacterial colony count in 85–100% water after 24 h. There was no significant decrease in bacterial growth in up to 95% water, suggesting that the presence of water did not impact the bacteria or their ability to use the nutrients supplied to them by the LB media present in the solution. For the 100% water condition, bacterial growth was reduced. However, these bacteria were viable after water exposure, as demonstrated by LB agar colony enumeration. *P. aeruginosa* was less affected by the presence of water than *S. aureus*. Water activity is defined as the ratio of the vapor pressure of water to that of pure water, which can be used to measure water that is available to be reacted with or attached to other materials. Higher water activity can support more microorganisms, with bacteria requiring water activity levels of at least 0.91 to grow.[42] Distilled water has a water activity of 1. This is based on the fact that Milli-Q water has a water activity of >0.91, as bacteria do not proliferate well but do survive in this form of water.[43] We speculate that water forced the cells into a low or zero growth rate due to a lack of nutrients. The ability of water to preserve pathogens for long-term storage (>30 weeks) suggests that water alone cannot affect the survival and viability of some bacteria.[44]

Next, the impact of silk solution concentrations on *S. aureus* and *P. aeruginosa* colony count was assessed (**Figure 3**). A silk content of 1–5% w/v affected bacterial growth significantly; both *S. aureus* and *P. aeruginosa* had a significant reduction in the number of bacterial colonies at all concentrations after a 24 h exposure when compared to the LB control ($p = <0.0001$ at all silk concentrations). Compared to the spiked 100% water control, all silk solution samples showed a significant reduction in bacterial colony numbers with both *S. aureus* and *P. aeruginosa* ($p = 0.0060$ and 0.0821, respectively). No *S. aureus* colonies were formed at 3% w/v silk solution and above, whereas *P. aeruginosa* did form a few colonies at 3% w/v but no growth occurred at 4% w/v and above.

The impact of sodium carbonate solution was also assessed on bacterial growth. We found that at concentrations below 0.5 mM, *S. aureus* and *P. aeruginosa* were able to survive (Figure S3B, Supporting Information) after incubation for 24 h. As silk solution was found to obtain ≈50 μM sodium carbonate, we propose that the bactericidal effects of silk could not be fully attributed to the presence of sodium carbonate. The numbers of both bacteria were reduced as sodium carbonate concentration increased, indicating a correlation between survival of bacteria and the presence of sodium carbonate.

Bacteria exposed to 100% v/v water overnight remained viable; this was in contrast to the observation for aqueous silk solution samples, where the bacteria had significantly lower viability. When the silk content was increased above 3% w/v in the aqueous solution, no colonies were detected. This suggests that the silk fibroin solution itself impacted bacterial viability, which is attributable to the alteration of water activity due to the addition of silk fibroin. Silk may decrease the amount of "free" water, reducing the water activity below the threshold and allowing the solution to become self-preserving, as supported by previously obtained sterility results.[45,46] Silkworms produce antimicrobial peptides (AMPs), such as cecropins, which effectively protect gram-positive and gram-negative bacteria from infection.[47] Silk fibroin has a five-amino-acid block "glycine-alanine-glycine-alanine-glycine-serine" (GAGAGS) sequence, which can assemble into β sheets in crystalline form but remains open in amorphous form, such as in aqueous solution. Glycine inhibits the growth of some bacteria and has been used as a non-specific antiseptic agent[48]; thus, the structure of silk could exhibit similar antiseptic effects on the bacteria in solution.

2.5. Performance of Silk Fibroin-Functionalized Wound Pad

As silk does not support growth in the absence of nutrients, we next determined its ability to act as an antimicrobial agent when applied topically to a bacterial suspension. The bacterial inhibition of the silk solution was determined by monitoring the bacterial growth of a silk-functionalized wound pad by exposing it to both LB agar and simulated wound fluid (SWF) agar (**Figure 4**). After wound pad removal, bacterial growth was observed on both types of agar. *S. aureus* formed light-colored spherical colonies when cultured on agar plates. On the LB agar plates, the dry sterile wound pad left a growth pattern. The wound pad pores were visible with no growth, implying that the wound pad aided growth on the agar when in direct contact with the surface. When the pad was soaked in 70% v/v ethanol, some growth was still observed at the pad borders, and some cloudiness was left beneath the pad. This suggests that the bacteria in contact with the wound pad surface could not grow, but the moisture may have enabled them to migrate and grow elsewhere. The silk-soaked pad left clearly defined growth borders around the wound pad. On simulated wound fluid agar plates, we observed a similar pattern, with clearly borders around the silk-soaked wound pad, with less defined borders in the ethanol-soaked pad and the dry sterile pad. Therefore, we speculate that the silk solution displaced the bacteria around the wound pad. Bacteria may not grow directly in the presence of silk but are not killed; instead, they grow on the enriched agar medium around the pad. *P. aeruginosa* formed less-

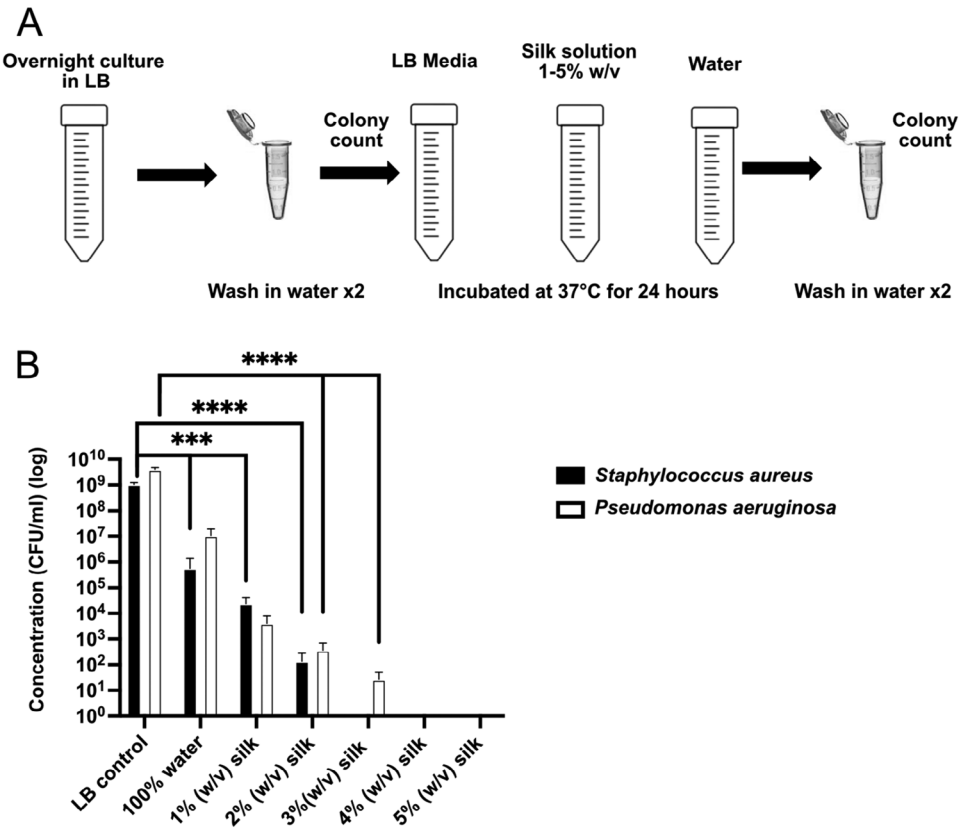


Figure 3. Minimum bactericidal and inhibitory concentrations of silk fibroin. A) Testing strategy. Overnight cultures of *S. aureus* and *P. aeruginosa* were incubated in LB media before being centrifuged at 12 100 × g for 5 min and the supernatant removed. This was repeated with water to wash the cells. The cells were then resuspended in LB media, water or aqueous silk solution and incubated at 37 °C, 150 rpm in an orbital incubator overnight. The cells were again washed through centrifugation and with water, as before. Colony counting was performed before the cells were suspended in the solution and after the final wash to determine viability. B) Minimum bactericidal concentration of silk fibroin aqueous solution. Colonies present after 24 h incubation in each pure silk spiked solution concentration ($n = 3$, ± SD). Data analysis evaluated by two-way ANOVA followed by Šídák's multiple comparisons test. Asterisks denote statistical significance determined using post-hoc tests as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

defined colonies, with green pyocyanin readily visible around the colonies. On the LB agar plates, the sterile dry pad left a clear pattern of contact with the wound pad. The ethanol-soaked pad left a visible rough border of growth, while the silk-soaked pad left clear borders where growth did not occur on the agar plates. On simulated wound fluid agar plates, a similar growth pattern was observed, with borders around the ethanol-and silk-soaked wound pads and a visible pattern where the dry pad was previously placed. This finding is similar to the results obtained for the interaction with *S. aureus*, indicating that these strains have similar responses to a silk solution and an ethanol solution. Silk solutions formulated as eye drops have been used as a dry eye treatment.[12] and as a topical treatment for diabetic wounds,[49] confirming the potential of silk for future biomedical uses. Silk, with its inherent sterility and antimicrobial effects, could therefore have potential for new applications in wound care.

The bacterial count of the wound pads after incubation in LB media for 15 min showed that when inoculated with *S. aureus*, the wound pad containing 70% v/v ethanol had significantly less bacterial growth than both the sterile dry pad and the silk solution-soaked pad. In contrast, *P. aeruginosa* showed no significant difference in any of the wound pads. These results, combined with the images of the dressing borders, suggest that, although the silk solution does not increase the bacterial growth of *S. aureus* or *P. aeruginosa*, it also does not reduce it significantly. Thus, the silk solution does not directly kill bacteria, as they grow when nutrients are introduced into their environment. These results are interesting, as pure aqueous silk shows sterility when stored over time, and when a pure silk solution is inoculated with pathogens directly, but used in addition to nutrient agar, bacterial growth occurs. This indicates that silk is bacteriostatic and not directly bactericidal.

Ethanol solution is a common antibacterial agent used to destroy pathogens on surfaces.[50] The ethanol-soaked wound pad disrupted bacterial growth better than the silk solution-soaked pad, which performed better than the dry pad at restricting growth under the pad on the agar for *S. aureus* (Figure 4b). The silk solution did not interrupt growth when in direct contact with the bacterial surface, because some growth was still observed under the wound pad around the edge, but the colonies found in and around the pad were not fewer than those observed under dry pad. This indicates that, in the presence of nutrients, the antibacterial effects of silk are limited. The presence of a solution can also enable bacteria migration ultimately allowing

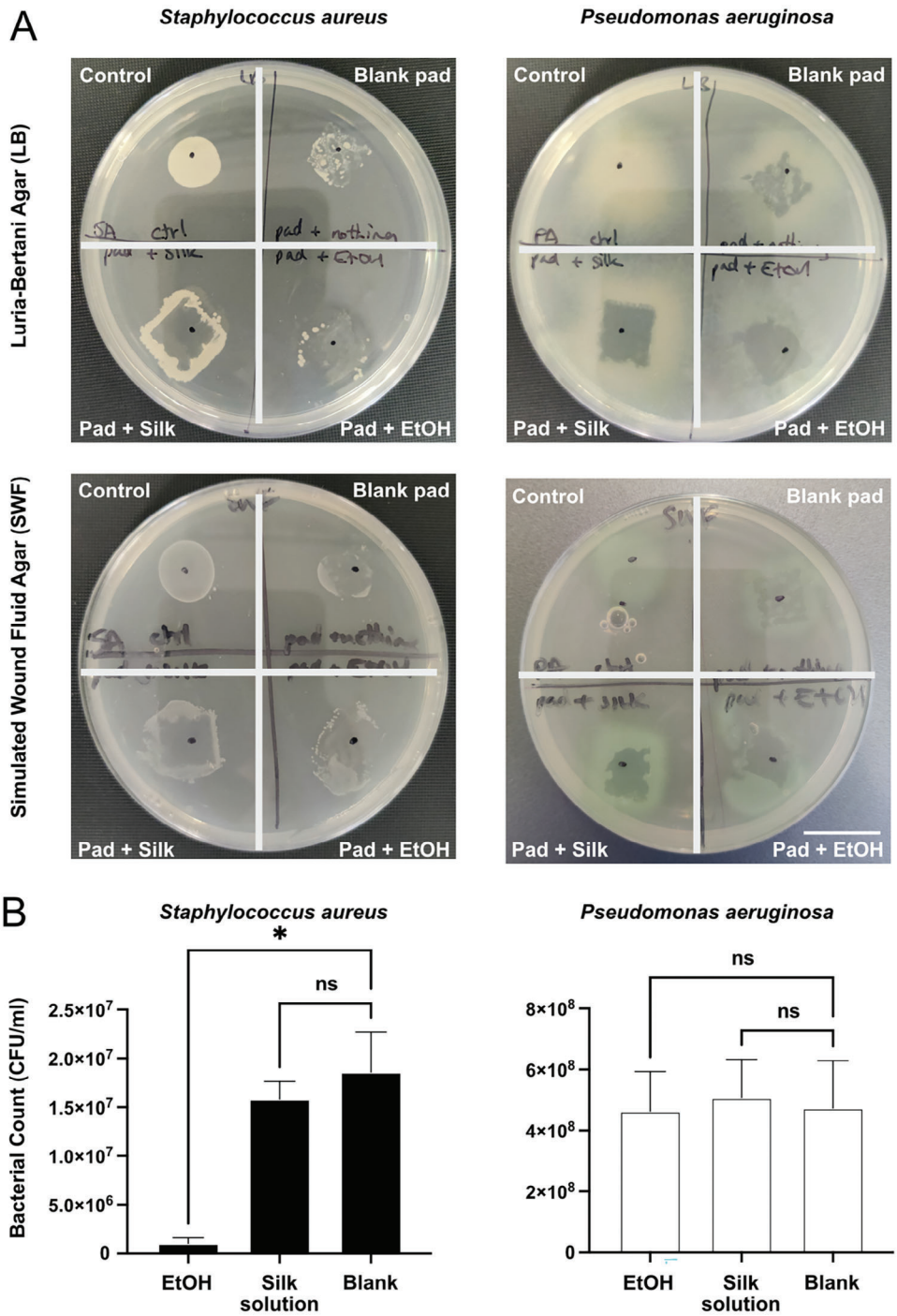


Figure 4. Simulated wound environment and the performance of silk-functionalized wound pad. A) Wound pad dry (control) or soaked in ethanol or silk removed to show growth on LB agar and simulated wound fluid agar. Scale bar 2 cm. B) *S. aureus* and *P. aeruginosa* wound pads incubated in LB media and colonies counted. ($n = 3$, ± SD). Data analysis evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks denote statistical significance determined using post-hoc tests as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

them to navigate away from the antibacterial agent to grow and thrive elsewhere. Antibacterial effects of *Bombyx mori* silk have been postulated because the silk cocoon, and the raw silk fiber, demonstrate antibacterial effects.[51] This property has been attributed to seroins and sericin that are present in raw silk.[52] Sericin has shown high antibacterial activity.[18,53,54] However, Kaur and coworkers[18,19] showed that pure silk fibroin and fibers lacked antibacterial properties against *Escherichia coli* contrasting earlier studies. This discrepancy was attributed to residual processing chemicals present in earlier studies.

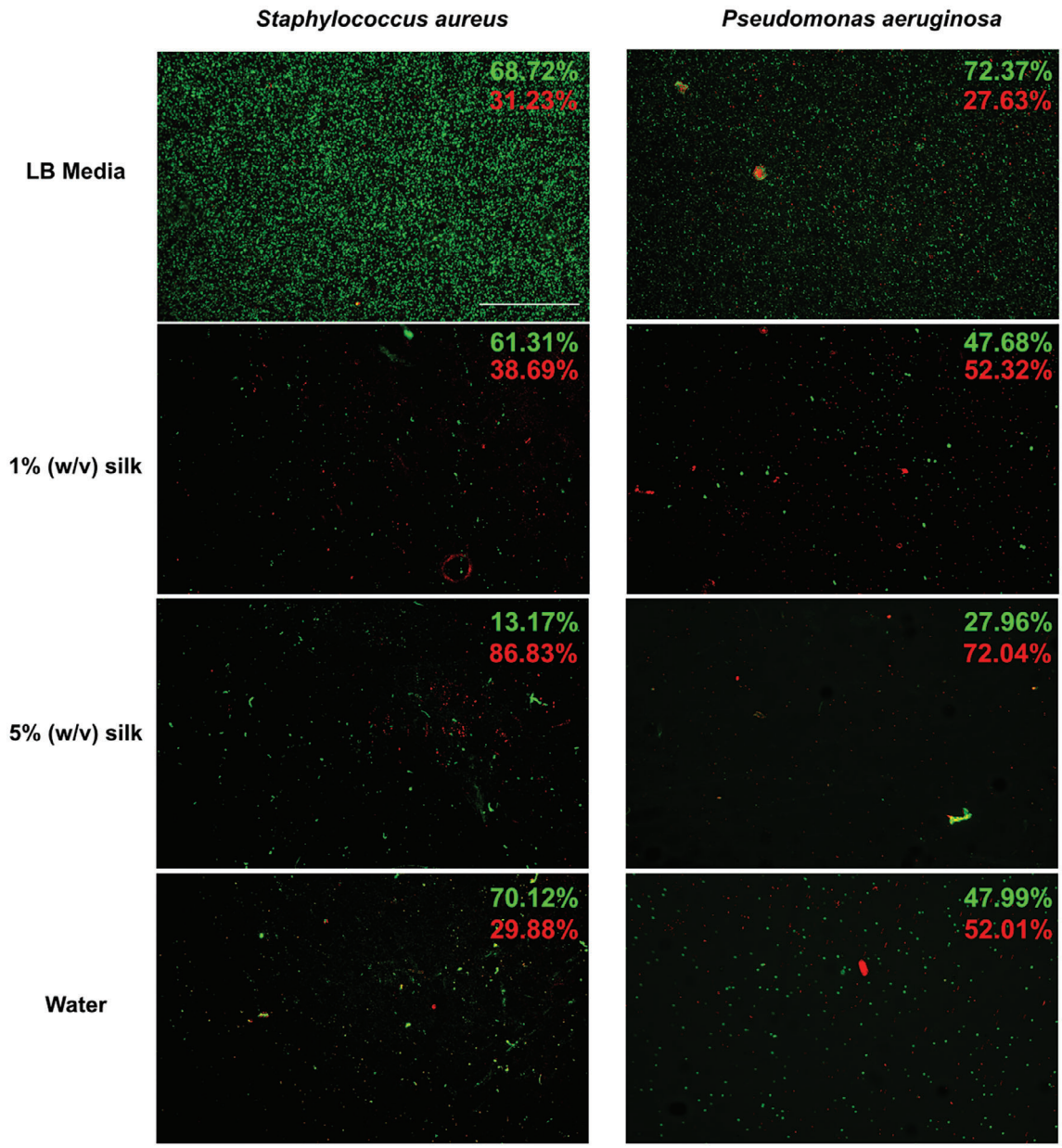


Figure 5. Impact of liquid silk on bacterial cell viability. Live/dead fluorescence staining. Green and red indicate live and dead cells, respectively. (Left) S. aureus in LB media, 1% w/v silk, 5% w/v silk and water. (Right) *P. aeruginosa* in LB media, 1% w/v silk, 5% w/v silk and water. Scale bar 200 µm. Numerical result $n = 3$.

2.6. Bacterial Viability

Bacterial viability after exposure to the silk fibroin solution was measured through live/dead imaging (**Figure** 5). A reduction in the number of live cells was observed for 100% water, 1% w/v aqueous silk solution and 5% w/v aqueous silk solution for both *S. aureus* and *P. aeruginosa*. The percentage of dead cells increased with the percentage of silk fibroin. The percentages of live *S. aureus* cells were 69% in the LB media and 70% in water. However, 61% and 13% of the cells were alive in 1% and 5% w/v silk solutions, respectively. Similar trends were observed for *P. aeruginosa* in LB media with 72% live cells, decreasing to 48% in 100% water and 48% and 28% in 1% and 5% w/v silk solutions, respectively. To confirm the viability of the stained cells, in parallel studies, samples were streaked onto LB agar. No growth occurred for either bacterial strain when exposed to a 5% w/v silk solution, while some growth occurred for the 1% w/v silk solution. This study also demonstrates that, in the presence of silk (e.g. 5% w/v), some viable bacterial cells still exist (albeit at much lower levels than the controls). However, when these silk-exposed cells were subsequently cultured on LB agar, no colonies were detected. Overall, these results suggest that some bacteria still had an intact plasma membrane after exposure to soluble silk but were unable to grow and ultimately survive,

even in favorable growth conditions. Pelegrini et al.[55] identified glycine-rich plant defense peptides for antibacterial purposes, determining that the interaction with the lipid layer of the bacterial cell wall causes cell death. A silk fibroin heavy chain contains 46% glycine[56]; however, the silk fibroin molecule also contains amorphous regions in the heavy chain, which provides flexibility and mobility to the molecule. We speculated that this allows the glycine-rich silk fibroin regions to have their antibacterial effect.

Therefore, we examined the potential of liquid silk to interact with the bacterial plasma membrane. The zeta potentials of *S. aureus* and *P. aeruginosa* cells were measured after incubation in water, LB media and silk solutions (Figure S5, Supporting Information). Neither bacterial strain showed any significant difference in zeta potential after incubation in water or LB media. Tests of the supernatant from the cultures spiked with silk also showed no significant differences in the zeta potentials. However, a significant difference was determined for bacteria incubated with either 1% (w/v) or 5% (w/v) silk fibroin (*S. aureus*: $p = 0.0388$; *P. aeruginosa*: $p = 0.0009$). This finding, in turn, suggested that an alteration of the cell surface charge due to the presence of silk fibroin ultimately impacted the ability of both bacterial species to proliferate. Interestingly, Halder et al.[57] reported that an alteration of the zeta potential can influence membrane permeability and ultimately trigger cell death (*E. coli* and *S. aureus*). Silk has an overall net negative charge; therefore, we believe that a charge–charge interaction between the plasma membrane and silk is unlikely, although the exact mechanism by which silk impedes bacterial growth remains to be established.

3. Conclusion

A silk fibroin aqueous solution has inherent antimicrobial effects. A silk solution with ≥4% w/v silk fibroin could inactivate *S. aureus* and *P. aeruginosa* contaminations. The silk solution did not support bacterial growth or survival. This opens up the possibility of using aqueous silk in biomedical applications. Importantly, liquid silk stored prior to processing is unlikely to experience microbial spoilage. This simplifies the handling and processing of liquid silk. However, this study also shows when liquid silk was applied to a simulated infected wound in the presence of nutrients, the antibacterial effects of the silk solution are not as effective. Overall, these results indicate that liquid silk is unlikely to spoil during storage but terminal sterilization of the final product is recommended.

4. Experimental Section

Silk Solution Preparation and Conductivity Assessment: The silk solution was prepared as detailed previously.[58,59] Briefly, *Bombyx mori* silk cocoons were cut into 5 × 5 mm pieces, and 5 g samples were degummed in 0.02 M Na_2CO_3 solution (2L) for 30 min. The purified silk fibroin was rinsed three times for 20 min in Milli-Q ultrapure water and then stretched and left to air-dry in a fume hood (airflow 0.5 m s^{-1}) overnight at room temperature. The dried silk fibroin fibers were then packed into a beaker and dissolved in a 9.3 M LiBr solution at 60 °C for up to 4 h. The resulting solution was dialyzed (MW cutoff 3500 g mol^{-1}, Thermo Scientific, Waltham, MA) for 48 h in Milli-Q water to remove the LiBr salt. Salt removal was monitored by taking conductivity measurements of the dialysate at 20–21.5 °C (Haiqingxin Shenzhen Purification Technology Co. Ltd, China). At the end of the dialysis procedure, the resulting silk fibroin solution was cleared by centrifuging twice at 9418 × g and 5 °C for 20 min. The silk fibroin content was determined gravimetrically. The silk fibroin solution was stored at 4 °C until use unless otherwise stated.

Silk Secondary Structure Analysis by Fourier Transform Infrared Spectroscopy: The secondary structures of silk samples were determined by Fourier transform infrared (FTIR) spectroscopy (Tensor II Bench ATR IR, Bruker, MA, USA). Each FTIR measurement was run for 128 scans at a 4 cm^{-1} resolution in absorption mode over the wavenumber range of 400–4000 cm^{-1}. Reference samples with low beta sheet content were prepared by air-drying 5% w/v silk fibroin solution to form a water-soluble film. Aqueous 1 and 5% w/v silk fibroin samples were incubated in a rocker-shaker at 150 rpm overnight at 37 °C, and then collected and air-dried into films. All FTIR data were deconvoluted as described previously.[60]

Sterility Testing: A silk solution (5% w/v) was divided into different treatment and storage conditions. The samples were either filter sterilized (0.22 µm Millex PES membrane, Merck Millipore Ltd, Cork, Ireland) or left unsterilized. The silk samples were then divided for storage at either 4 °C, room temperature (25 °C) or 37 °C for 14 days in sealed aseptic containers.

The sterility of the silk solutions was tested by two methods. For the first method, tryptic soy broth (TSB) was produced according to manufacturer's instructions by dissolving 40 g tryptone soya broth powder (Oxoid, Hampshire, England) in 1 L Milli-Q H_2O and autoclaving for 20 min at 121 °C. Next, 1 mL of silk solution (freshly prepared or stored for 14 days) was added to 9 mL TSB, and incubated at either room temperature or 37 °C for 14 days, and then imaged. The turbidity of the solutions was visually assessed to determine the presence of contamination. Control samples were also prepared by spiking TSB with known concentrations of *S. aureus* and *P. aeruginosa* and imaging before and after incubation for 24 h. Bacterial colonies were then counted after 24 h to assess growth rate and turbidity detection.

The second method used blood and chocolate agar plates. Briefly, blood and chocolate agar was prepared by dissolving a 40 g blood agar base (Fluka analytical, Buchs, Switzerland) in 1 L Milli-Q H_2O and autoclaved for 20 min at 121 °C. The blood and chocolate agar were prepared following the manufacturer's instructions with defibrinated horse blood and stored at 4 °C until use. The silk solution was tested by streaking 10 µL samples of each condition onto both blood and chocolate agar plates and storing them either in aerobic or anaerobic conditions at 37 °C for 72 h and imaging them after visually assessing growth.

Culture Preparation: The bacteria used for this study were *S. aureus* (NCTC 8325) and *P. aeruginosa* (PA 14) grown in Lennox LB medium, prepared with 10 g tryptone, 5 g NaCl, and 5 g yeast extract in 1 L Milli-Q H_2O autoclaved for 20 min at 121 °C. LB agar plates were prepared by the addition of 20 g agar to LB medium before autoclaving, and 20 mL was poured into 9 cm diameter Petri dishes and then cooled. Stock cultures were produced by inoculating 10 mL of LB medium with a single colony and incubating overnight at 37 °C in a shaker incubator at 150 rpm (ES-20, Grant instruments, Cambridge, UK).

Bacterial Growth in the Presence of Water: Bacterial growth and survival were assessed in the presence of water. Briefly, overnight cultures of *S. aureus* and *P. aeruginosa* were prepared as described above. The samples were centrifuged at 12 100 × g for 5 min and the supernatant was removed. The bacterial pellet was resuspended in sterile water and centrifuged again. This washing step was repeated twice. A sample of bacteria suspended in water was taken for colony counting, by performing serial dilution and plating 10 × 10 µL drops on an agar plate and incubating overnight. The colonies were counted and summed across the ten drops, and the total concentration was calculated. All samples were then spiked with bacteria in the order of 10^8 CFU mL^{-1}. The bacteria were then centrifuged once more and then suspended in either 0%, 75%, 85%, 95%, or 100% sterile water with LB medium. The samples were incubated for 24 h at 37 °C in a shaker incubator (ES-20, Grant Instruments, Cambridge, UK). A 10 µL sample of each inoculated mixture was streaked onto an LB agar plate and incubated overnight. The colonies were counted at time 0 and after 24 h of growth.

Minimum Bactericidal Concentration of Silk: The minimum bactericidal concentration (MBC) of silk was determined in a similar method to that used for the determination of the water MIC. Briefly, overnight cultures of *S. aureus* and *P. aeruginosa* were prepared as described above. Bacterial samples were prepared as described in Section 2.5. All samples were spiked with bacteria in the order of 10^8 CFU mL^{-1}. The bacteria were suspended in either 1%, 3%, 4%, or 5% w/v filter-sterilized aqueous silk solution and incubated for 24 h at 37 °C in a shaker-incubator (ES-20, Grant Instruments, Cambridge, UK). A 10 µL sample was taken from each inoculated silk mixture, streaked onto an LB agar plate, and incubated overnight. Colony counting was performed as before and the colonies were counted at time 0 and after 24 h of growth.

Simulated Wound Dressing: The antibacterial effect of the silk solutions was tested in the presence of bacterial nutrients. Briefly, simulated wound fluid agar was prepared by adding heat-inactivated foetal bovine serum (Biosera, East Sussex, UK) to a physiological salt solution at a ratio of 1:1 at 56 °C. The physiological salt solution was prepared with 142 mM NaCl, 2.5 mM $CaCl_2$ and dH_2O and autoclaved. The LB agar plates were prepared as before.

To mimic the application of a dressing, the wound pad of a fabric plaster (Elastoplast, Beiersdorf, Hamburg, Germany) was cut and soaked in either 5% w/v silk solution or 70% v/v ethanol. Overnight cultures of *S. aureus* or *P. aeruginosa* were prepared as before. These were then diluted to a final concentration of 10^6 CFU mL^{-1} when 100 µL solution was placed in the center of four quadrants of the agar plates. Freshly soaked wound pads were placed over the bacterial suspension and incubated at 37 °C overnight. The wound pad was then removed, and the plates were imaged. Infection levels were determined by placing the wound pad in LB broth for 15 min and then performing colony counting (detailed above).

Bacterial Viability: Bacterial viability was measured with a live/dead Baclight staining kit (ThermoFisher Scientific, UK). Briefly, overnight cultures were prepared and exposed to silk (detailed above). Bacterial samples were prepared as described in Section 2.5. The samples were then resuspended in either 4 mL of LB medium, water, 1% w/v silk solution, or 5% w/v silk solution and incubated overnight at 37 °C at 151 rpm. Samples (1 mL) were collected the following day, centrifuged at 12 100 × g for 5 min, washed once with water, and resuspended in sterile 0.85% w/v NaCl. The viability kit was then used according to the manufacturer's protocol. Samples were added to microscope slides and imaged using an epifluorescence microscope (Nikon Eclipse E600) with excitation/emission 470/515 nm (green filter, viable cells) and 530/635 (red filter, dead cells). Images were processed and analyzed with ImageJ software v1.53f51 (National Institutes of Health, USA).

Sodium Carbonate Detection and Bactericidal Effects: Sodium carbonate was detected with a sodium ion selective electrode (perfectION, Mettler Toledo AG, Switzerland). Briefly, a freshly prepared 0.02 M Na_2CO_3 solution was measured before and after silk degumming. Next, the degummed silk was rinsed three times in 1 L water and samples of wash medium were taken for sodium detection. The silk fibroin solution was dialyzed and prepared as detailed above. The sodium content of the final silk fibroin solution (5% w/v) was also recorded. Based on these measurements, sodium carbonate reference solutions were prepared to determine the minimum bactericidal concentration.

Zeta Potential Measurements: The zeta potential of the bacteria was measured in the presence and absence of liquid silk fibroin. Briefly, bacterial samples were exposed overnight to liquid silk fibroin (1% w/v and 5% w/v), water or LB culture medium (detailed above 2.6). The surface charge of the samples was then measured with a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK).

Silk Fibroin Pyrene Study: The silk fibroin solution conformation was monitored using pyrene labeling.[37] Briefly, pyrene was dissolved in ethanol and diluted with water to a working stock of 5.5×10^{-5} M pyrene in 20% ethanol. This working stock was added to the silk fibroin solution at a solution ratio of 1:100 (v/v) to produce a final pyrene concentration of 5.5×10^{-7} M pyrene (0.02% ethanol). The fluorescence emission spectra of 1% to 5% (w/v) silk fibroin samples were measured using a spectrofluorophotometer (RF-5301 Shimadzu Europe, Duisburg, Germany) over a wavelength range of 350–550 nm, with an excitation wavelength set at 336 nm. The ratio of the fluorescence emission (the Py value) was calculated by taking the emission at 375 nm (band I) and 385 nm (band III) and calculating the band I to band III ratio.

Statistical Analyses and Data Sets: Statistical analyses were carried out using Origin Pro 2018 (Northampton, Massachusetts, USA) and GraphPad Prism v.9.1.1 (San Diego, CA, USA). Normality and homogeneity of variances were assumed. One-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test, was conducted between multiple groups, or two-way analysis of variance (ANOVA) was conducted, followed by Tukey's multiple comparisons. Asterisks denote statistical significance determined using post-hoc tests as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Statistical analyses are noted in figure legends. All data are presented as mean values ± standard deviation, and the number of independent experiments (n) is noted in each figure legend.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

G.E. designed, collected, analyzed, and interpreted the data and generated the manuscript draft. A.H. provided support and training for data collection and analysis. S.D. provided support and expertise with ion detection and analysis. P.C. and F.P.S supervised and conceived the study. F.P.S. and P.C. content edited the manuscript.

Data Availability Statement

The data that support the findings of this study are openly available in the Strathclyde PURE Portal at DOI: https://10.15129/8ac55526-a7cc-4363-bc4b-38a9c9b146d0

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